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RESPONSE UNDER 37 C.F.R. 1.116
EXPEDITED PROCEDURE
EXAMINING GROUP 1634

PATENT

S/N 09/125,953

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	FODSTAD et al.	Examiner:	Sisson, B.
Serial No.:	09/125,953	Group Art Unit:	1634
Filed:	December 10, 1998	Docket No.:	7885.56USWO
Title:	IMMUNO-MAGNETIC CELL SEPARATION USED IN IDENTIFICATION OF GENES ASSOCIATED WITH SITE-PREFERENCED CANCER METASTASIS FORMATION		

OFFICIAL

CERTIFICATE UNDER 37 CFR 1.6: The undersigned hereby certifies that this correspondence is being transmitted via facsimile to: TC1600, Commissioner for Patents, Washington, D.C. 20231 on August 12, 2002.
By: Michele Quaranto
Name: Michele Quaranto

SUBMISSION OF SIGNED DECLARATION

Box AF
Commissioner for Patents
Washington, D.C. 20231

VIA FACSIMILE
TC1600
703-872-9307

Dear Sir:

In response to the Office Action mailed February 27, 2002, and further to our response of July 26, 2002, Applicants enclose herewith a signed copy of the Declaration of Oystein Fodstad that was previously submitted unsigned. Applicants respectfully request consideration of the declaration.

Respectfully submitted,

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Date: 8/12/02

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PATENT TRADEMARK OFFICE

PATENT

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DECLARATION OF ØYSTEIN FODSTAD

I, Øystein Fodstad, M.D., Ph.D. am an inventor of the above-referenced application. I am head of the Department of Tumor Biology and Director, Institute for Cancer Research Council, at The Norwegian Radium Hospital, University of Oslo and have extensive experience in the biotechnical arts, including antibody production and separation. I have read and understood the Official Actions dated July 31, 2001 and February 27, 2002 issued by Examiner Sisson.

My invention is a method for identifying genes differentially expressed between cells isolated from different tissues, the method comprising:

(A) detecting target cells from a first and a second tissue;

(B) obtaining nearly 100% specific target cells by repeatedly immunomagnetically isolating, *in vitro*, said first and second tissue target cells;

(C) determining levels of mRNA expression within said first and second tissue target cells;

(D) comparing the levels of mRNA expression in said first and second tissue target cells; and

(E) based upon the comparison in step D, identifying the genes differentially expressed between said first and second tissue target cells, wherein at least one of said first and second tissue target cells are tumor cells, in order to recognize previously unknown genes possibly involved in determining metastatic characteristics of cancer cells.

One advantage of my invention is that it allows for easier identification of genes differentially expressed between primary tumors and metastatic tumors. Identification and study of such genes could prove invaluable in determining, for example, the mechanisms associated with metastasis. Prior to my invention, it was not thought possible to perform meaningful gene closing experiments on specimens of solid tumors and metastases for the purposes of identifying genes with site-specific expression. This is because it was thought impossible to separate tumor cells from normal cells without first culturing the cells and performing manipulations to get rid of the normal cells. However, the culture conditions resulted in changes in gene expression relative to *in vivo* expression. These changes in gene expression rendered gene cloning experiments for the identification of genes involved in the metastatic process meaningless when culturing was performed to isolate tumor cells from surrounding normal cells. Yet without the isolation of tumor cells from surrounding normal cells, the background gene expression of the normal cells posed similar problems. My invention overcomes these problems by providing a method by which target cells can be separated form a cell population in order to identify the gene sequences from the target cells in a specific cell population environment.

At the time of my invention, a scientist in the field of molecular and cellular biology would have known how to perform the following method steps individually:

1. Immunomagnetically isolate, in vitro, cells obtained in vivo;
2. Determine levels of mRNA in cells; and
3. Compare levels of mRNA between cells and to identify whether the mRNA is differentially expressed.

IMMUNOMAGNETIC ISOLATION, *IN VITRO*, OF CELLS OBTAINED *IN VIVO*

At the time of my invention, techniques for immunomagnetically isolating cells were known. For example, page 4 of the application refers to two PCT patent applications, WO94/07139 and WO95/24648, which teach the ability to (A) detect target cells from a tissue and (B) obtain nearly 100% specific target cells by repeatedly immunomagnetically isolating, *in vitro*, the target cells.

Briefly, these results can be achieved by a method which involves binding of monoclonal antibodies that specifically recognize antigens present on tumor cells, and not on the normal cells

in question, or for other purposes to specified subpopulations of normal cells, to magnetic particles, either directly or to beads first covered with antibodies specifically recognizing the immunologic isotype of the respective antibodies that bind to the tumor cells. Such antibodies may be of the IgG type or may be a fragment of IgG or IgM. The targeting antibodies may be attached directly to the magnetic particles, or the binding to cells can take place by using beads coated with antibodies specifically recognizing the Fc portion of the said individual antibodies already bound to the target cells. The antibody coated magnetic beads are mixed with the suspension of cells to be examined, incubated for 30 minutes at 0-10°C, preferably 4 °C under gentle rotation. Samples of the cell suspension are then transferred to a cell counting chamber, and the fraction of cells with attached beads relative to the total number of cells is determined under light microscopy. The visualization of target cell-particle complexes, the rosettes, makes it simple to directly spot the target cells with up to 100% specificity.

In WO95/24648, the above described method is improved by further transferring the suspension of target cell-particle complexes to a cell filtering device, and after performing the filtration the target cell-particle rosettes can be viewed microscopically on the filter membrane or grown in a physiological base culture medium, e.g. without separation the target cells from the particles, to be characterized for the presence of specific biochemical and biological features.

Wang, et al. (Pathology Oncology Research 1995) teach an immunomagnetic procedure for isolating specific cells from a mixed cell population using antibody-coated beads. Thus, at the time the invention was made, one of ordinary skill in the art would know how to isolate cells using antibody-coated beads.

DETERMINATION OF LEVELS OF MRNA WITHIN A TISSUE

At the time of my invention, one would have known how to determine levels of RNA within a tissue. Several well known techniques, such as reverse transcriptase PCR (RT PCR), in situ hybridization, RNAse protection assays, and Northern blots are available for such purposes. A search of the PubMed Database at http://www.ncbi.nlm.nih.gov/PubMedOld/medline.html for journal articles published in the year 1995 describing RT PCR resulted in 1,315 hits. Similar searches for publications in 1995 were performed for in situ hybridization, RNAse protection, and Northern blot. The results are presented in table 1.

Table 1. Number of publications in PubMed database disclosing methods and techniques associated determinations of RNA levels

Search Term	Hits for year 1995
RT PCR	1,315
In Situ Hybridization	4,878
RNAse Protection	395
Northern Blot	2,007

The number of hits obtained in Table 1 should be considered as a floor for the actual number of journal articles disclosing these techniques as the search terms entered do not cover all possible deviations in nomenclature. Regardless of inconsistency in nomenclature, more than 7,000 publications disclosed well-known techniques for determining RNA levels in tissues in 1995 alone.

Determining the level of RNA in a tissue was well within the ability of the skilled artisan at the time of filing of the above-referenced application. However, a brief general overview of RT PCR and Northern blot techniques may provide useful for discussion of the appended references.

In an RT PCR assay, cDNA is generated by incubating RNA, which has been isolated by known techniques, with reverse transcriptase enzyme. The cDNA can then be amplified by PCR using oligonucleotide primers that hybridize to an upstream and a downstream portion of a cDNA molecule of interest (which corresponds to the RNA species of interest). PCR amplifies, or produces, in an exponential fashion, copies of the cDNA molecule of interest between the areas in which the primers hybridize. The amplified DNA can be quantified in a visual or by an automated process. A "housekeeping" gene can be amplified to correct for unequal amounts of starting material. A housekeeping gene is a gene that is generally present at similar levels in all cells. An example of a housekeeping gene is β-actin. This correction is typically achieved by dividing the amount of signal generated from the amplified region of interest by the signal generated from the amplified portion of the housekeeping gene.

In Northern blot analysis, RNA is isolated from a tissue by known techniques, separated by apparent molecular weight by applying a voltage differential across a gel into which the RNA is loaded, and either electrically or osmotically transferred to a membrane, such a polyvinyl

fluoride membrane. The membrane is then typically probed for the presence of an RNA species with an excess of a cDNA molecule capable of hybridizing to the RNA species of interest. The membrane is washed to remove unhybridized cDNA molecule. Attached to the cDNA molecule is a detectable label that can be exploited to quantify the number of RNA molecules of the species of interest to which the labeled cDNA molecule bound. Sometimes an RNA molecule, rather than a cDNA molecule is used as a probe. Additionally, a similar technique known as the dot blot can be used. In a dot blot assay, the RNA containing the species of interest is directly blotted onto the membrane.

In Northern blot analysis it is often desired to probe for a "housekeeping" RNA species in addition to the species of interest. A "housekeeping" RNA species is an RNA species that is generally present at similar levels in all cells. An example of a "housekeeping" RNA species is 18S rRNA. Probing for a housekeeping RNA species in addition to the RNA species of interest allows for corrections for uneven amounts of RNA loaded in various lanes of the gel. This correction is typically achieved by dividing the amount of signal generated from the probe to the RNA species of interest by the signal generated from the probe for the housekeeping RNA species. The corrected signal can be quantified visually or through an automated process.

As examples of specific references that disclose techniques for determining RNA levels in a tissue, please find enclosed Appendices 3 -6. Appendix 3 is Mælandsmo et al., Cyclin kinase inhibitor $p21^{WAF1/CIP1}$ in malignant melanoma, *American Journal of Pathology*, 149(6):1813-1822 (1996). This journal article discloses Northern blot analysis for detecting *WAF1/CIP1* RNA levels in malignant melanomas. Specifically, a *WAF1/CIP1* cDNA probe was used to probe total cellular RNA, with an 18S rRNA probe being used to correct for unequal loading. Appendix 4 is Mælandsmo et al., Reversal of the *in vivo* metastatic phenotype of human tumor cells by an anti-*CAPL* (*mts1*) ribozyme, *Cancer Research*, 56: 5490-5498 (1996). This journal article discloses the use or Northern blot analysis and RT-PCR to quantify the level of *CAPL* RNA. In the Northern blot analysis 18S RNA was used to correct for unequal loading after scanning an audioradiogram in a densitometer. In the RT PCR assay, β-actin was used to correct for differences in starting material. The quantification was based on a colorimetric assay. Appendix 6 is Deggerdal et al., Semiquantitative polymerase chain reaction for t(14;18) in follicular lymphomas: A colorimetric approach., *Laboratory Investigation*, 72(4): 411- 418 (1995). This journal article disclosed a method for quantifying translocation-positive cells.

While this reference does not teach the quantification of RNA from a tissue, one skilled in the art would recognize that the methods disclosed in this reference would be applicable to quantification of RNA by the additional initial step generating cDNA by using reverse transcriptase.

COMPARISON OF MRNA LEVELS BETWEEN TWO DIFFERENT TISSUES TO DETERMINE WHETHER MRNA IS DIFFERENTIALLY EXPRESSED

At the time of my invention, one would have known how to compare levels of RNA between two different tissues to determine whether mRNA was differentially expressed. Several well known techniques, such as differential display and subtractive hybridization were available for such purposes. A search of the PubMed Database at http://www.ncbi.nlm.nih.gov/PubMedOld/medline.html for journal articles published in the year 1995 describing differential display resulted in 213 hits. A similar search for publications in 1995 that described subtractive hybridization resulted in 54 hits. Clearly, these techniques were known and available to those interested determining whether mRNA is differentially expressed between two tissues.

Representative references teaching such techniques include:

1. Ebralidze et al., *Genes Dev.* 3: 1086-1093 (1989)
2. Liang and Pardee, Science 257:967-971 (1992)
3. Liang et al. CAncer Res. 52:6966-6968 (1992)
4. Rosol et al., Biotechniques 21:114-121 (1996)
5. Sobel, J. Natl. CAncer Inst. 82:267-276 (1990)
6. Lee et al., PNAS 88:2825-2829 (1991)
7. Sager, Curr. Opin. Cell Biol, 4:155-160 (1992)
8. Owens and Cohen, Cancer Metastasis Rev. 11:149-156 (1992)

EVIDENCE THAT TEACHINGS OF APPLICATION CAN BE APPLIED TO ACHIEVE MY INVENTION

My invention has been successfully used to isolate nucleic acid sequences potentially involved in metastasis. Three scientific journal articles showing successful use of my invention are attached to this declaration. First, Ree et al., "Expression of a novel factor in human breast cancer cells with metastatic potential," *Cancer Research* 59: 4675-4680 (1999), shows the

isolation of a nucleic acid sequences that may play a role in metastasis. Human breast carcinoma cells were injected into the systemic circulation of immunodeficient rats. Resulting CNS metastatic tumors were isolated from the rats. Metastatic cells were isolated using immunomagnetic beads coated with an antibody that was reactive with human cells. The segregated cell population was compared with the injected cells by means of differential display analysis. Two nucleic acid molecules that may play a role in metastasis were identified. The biological mechanism of one of these nucleic acid molecules, com1, was studied in a second journal article (Bratland et al., Expression of a novel factor, com1, is regulated by 1,25-dihydroxyvitamin D3 in breast cancer cells, *Cancer Research* 60: 5578-5583, 2000) revealing potential insights into the metastatic process. In a third journal article (Ree et al., "Differential display analysis of breast carcinoma cells enriched by immunomagnetic target cell selection - gene expression profiles in bone marrow target cells", *Int. J. Cancer*, 97:28-33, 2002) additional candidate nucleic acid molecules were identified.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such false statements may jeopardize the validity of the application or any patent issued thereon.

Date: August 5, 2002

Øystein Fodstad